



## Aqueous two-phase systems: A simple methodology to obtain mixtures enriched in main toxins of *Bothrops alternatus* venom



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### ABSTRACT

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and protease (P) are enzymes responsible of myotoxic, edematogenic and hemostasis disorder effects observed in the envenomation by *Bothrops alternatus* pitviper. Their partitioning coefficient (K<sub>p</sub>) in different polyethyleneglycol/potassium phosphate aqueous two-phase systems (ATPSs) was determined in order to both achieve a better understanding of the partitioning mechanism and define optimal conditions for toxin isolation. Polyethyleneglycols (PEGs) of molecular weights 1000; 3350; 6000 and 8000; different temperatures (5, 20 and 37 °C) and phase volume ratios of 0.5; 1 and 2 were assayed. PLA<sub>2</sub> partitioned preferentially to the top phase while P mainly distributed to the bottom phase. Either entropically- or enthalpically-driven mechanisms were involved in each case (PLA<sub>2</sub> and P). The aqueous two-phase system formed by PEG of MW 3350 (12.20% wt/wt) and KPi pH 7.0 (11.82% wt/wt) with a volume ratio of one and a load of 1.25 mg of venom/g of system showed to be the most efficient to recover both enzymes. It allowed obtaining the 72% of PLA<sub>2</sub> in the top phase with a purification factor of 2 and the 82% of P at the bottom phase simultaneously. A further adsorption batch step with DEAE-cellulose was used to remove satisfactorily the PEG from the top phase and recover the active PLA<sub>2</sub>.

The proposed methodology is simple, inexpensive, and only requires professionals trained in handling basic laboratory equipment. It could be easily adoptable by developing countries in which the snakebite accidents cause considerable morbidity and mortality.

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### 1. Introduction

Envenoming resulting from snakebites is considered by the World Health Organization one neglected tropical disease which represents an important worldwide public health concern, particularly in developing countries of Africa, Asia and Latin America [1]. The victims of snakebites are mostly young –agricultural workers and children–, therefore, the consequent economic impact is considerable. A timely administration of a specific antiserum has been demonstrated to be an effective tool; however, the poor access to health services and the scarcity of adequate antivenomits in the mentioned countries leads to poor

outcomes and considerable morbidity and mortality [2].

Snake venoms are complex mixtures of different biomolecules such as peptides, proteins and enzymes, some of which are responsible for their toxic effects [3,4]. Antivenoms are usually obtained by immunizing host animals –horses, rabbits, sheeps– with successive inoculations of increasing amounts of the whole lethal venom. Prolonged immunization plans are required to avoid damaging effects in animals and to obtain efficient antisera. An alternative strategy, that includes a pre-immunization of horse with phospholipase A<sub>2</sub> –one of the main toxic components of *Crotalus durissus terrificus*– has been proposed by Fusco et al. [5]. This protocol demonstrated to avoid both impairing animal health and improving the antiserum neutralizing efficacy. More recently, we observed that shorter immunizing protocols, milder toxic effects and antisera with high neutralizing ability were also obtained by inoculating animals with the whole venom enriched in

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its predominant toxins [6].

In this context, the availability of appropriate antigenic mixtures containing the main venom toxins associated with damaging or lethal effects is an important requirement in manufacturing more specific antibodies. In addition, it is a good starting point in developing vaccines [7] and sensitive immunoassays for snake venom detection in medical diagnostic [8].

The composition, immunogenicity and effects of venom vary intra and inter species, thus depending on the geographical distribution and the age of the specimens. Venom toxins to include in a venom pool used for animal immunization should be selected on the basis of the geographical region where the anti-venom is intended to be distributed [7].

The pitvipers inhabiting Central and South America belong to the genus *Bothrops*. Particularly, *Bothrops alternatus* is a species widespread in Brazil, Paraguay, Uruguay and Argentina, whose bites create severe local tissue damage, local and systemic hemorrhagic effects. Among its components, the phospholipases A<sub>2</sub> and the metalloproteases have been reported to play primary roles in myotoxic, edematogenic and hemorrhagic effects observed in the envenomation by this pitviper [9–12]. The complexity of the crude venom makes it difficult to separate mixture. A multistep separation consisting of gel filtration, ion-exchange and reversed phase high-pressure liquid chromatography is usually needed [13,14] to obtain an enzyme of desirable purity. This requires resources scarcely available in developing countries or rural regions such as advanced equipment—columns, pumps and matrix—and professionals trained in special techniques.

Liquid–liquid extraction with aqueous two-phase systems (ATPSs) has proved to be a powerful tool for separating and purifying mixtures of biomolecules [15,16]. Culture media and crude extracts can be directly loaded into the ATPSs without a previous centrifugation or filtration step [17]. This technique exhibits several advantages such as simplicity, short processing times and low cost [18]. Furthermore, this process does not require sophisticated equipment and its scale-up is easy and reliable [19]. The ATPSs have been used in a wide range of biotechnological applications such as the recovery of recombinant proteins in corn [20], the purification of human antibodies [21] and Plasmid DNA [22] and the extraction of trypsin and chymotrypsin from bovine pancreas [23,24].

Protein partitioning and selectivity of the ATPS extraction depends on factors such as the phase-forming salt and polymer, the volume ratio (Vr) of phases and the sample load. The relation between the partition coefficient (Kp) of a target biomolecule and the ATPS parameters must be evaluated previously to develop an appropriate extraction protocol.

The goal of this work was to explore those variables that affect the partitioning equilibrium of phospholipase A<sub>2</sub> and protease toxins present in *B. alternatus* venom in order to optimize the extraction and develop a strategy capable of recovering venom mixtures enriched in the mentioned toxins. These toxins represent more than 50% of total venom protein [25] and are responsible of the main local and systemic effects caused in snakebites victims. At the same time, this information could contribute to a better understanding of the mechanisms involved in protein partition in ATPSs.

## 2. Materials and methods

### 2.1. Chemicals

Polyethyleneglycols of average molecular masses 1000; 3350; 6000 and 8000 (PEG1000, PEG3350, PEG6000, PEG8000) were purchased from Sigma Chem. Co. and used without further purification. All other reagents were of analytical quality.

Pooled crude venom of *B. alternatus* pitvipers was obtained from the Serpentarium of the local zoo in Corrientes, Argentina. It was lyophilized and then, stored at –20 °C until being required. Before using, the venom was diluted with phosphate buffered saline solution (PBS) pH 7.2, the resultant suspension (50 mg lyophilized venom/mL PBS) being applied for studies.

### 2.2. Experimental

#### 2.2.1. Partition measurements

**2.2.1.1. Preparation of the aqueous two-phase systems.** Stock solutions of the phase components: potassium phosphate (KPi) pH 7.0 (30% w/w), solid PEG—of different molecular weights— and water were mixed in order to prepare the two-phase aqueous systems (total mass 20 g). The total system compositions selected from the binodal diagrams in literature [18] are shown in Table 1. After a thorough gentle mixing of the system components, low speed centrifugation was used to favour the phase separation. Phases were withdrawn and used to reconstitute several two-phase systems, in which the venom partitioning behaviour was evaluated.

**2.2.1.2. Determination of the partition coefficients (Kps) of phospholipase A<sub>2</sub> and protease.** Partitioning behaviour of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and protease (P) was analysed by dissolving a given amount of venom suspension (50 µL) into two-phase systems of total mass 2 g with different ratios of top/bottom phase-volumes (0.5; 1 and 2). After mixing by gentle inversion for 10 min and leaving it to settle for at least 60 min, each system was centrifuged at low speed for the two-phase separation. These experiments were carried out in graduated tubes in order to appreciate the top/bottom phase-volumes. No volume-changes were observed after partitioning. Appropriate aliquots from separated phases were taken and diluted conveniently to determine the content of PLA<sub>2</sub> and P through activity measurements. The content of total protein (TP) in each phase was also determined according to the description below.

The partition behaviour of PLA<sub>2</sub>, P and TP was evaluated by calculating the partition coefficient according to:

$$K_p = \frac{[\text{protein}]_{\text{top phase}} \cdot f_{\text{top}}}{[\text{protein}]_{\text{bottom phase}} \cdot f_{\text{bottom}}} \quad (1)$$

The [protein] was replaced by enzyme activity when calculating the partition coefficient of PLA<sub>2</sub> and P. A correction factor ( $f_{\text{top}}$ ,  $f_{\text{bottom}}$ ), which comprises the effect of the components of a given phase (top/bottom) on the activity measurements, was included. It was calculated as follows:

**Table 1**  
Total and phase compositions for PEG/Pi ATPSs.

Total composition (% wt/wt)		Top phase composition (%wt/wt)		Bottom phase composition (%wt/wt)	
KPi	PEG	KPi	PEG	KPi	PEG
PEG1000					
16.20	17.00	3.10	39.10	24.00	1.60
PEG3350					
11.82	12.20	5.56	23.90	17.41	1.30
PEG6000					
11.04	13.98	4.58	25.64	16.60	0.46
PEG8000					
10.90	15.00	3.10	33.00	15.59	1.60

$$f_{\text{top/bottom}} = \frac{\text{Act}_{\text{PBS}}}{\text{Act}_{\text{top/bottom}}} \quad (2)$$

where  $\text{Act}_{\text{PBS}}$  and  $\text{Act}_{\text{top/bottom}}$  are the activities measured in samples prepared by dissolving 50  $\mu\text{L}$  of venom suspension in 1 mL of either PBS or top/bottom phases.

Temperature was maintained constant at three temperatures (5, 20 and 37 °C) and controlled to within  $\pm 0.1$  °C by immersing the tubes in a thermostatic bath. All the measurements were carried out in triplicate.

**2.2.1.3. Thermodynamic characterization of the protein partitioning.** The thermodynamic functions  $\Delta G^\circ$ ,  $\Delta H^\circ$  and  $\Delta S^\circ$ , associated to the partitioning of  $\text{PLA}_2$  and P, were calculated according to classical equations as follows.

The free energy change ( $\Delta G^\circ$ ) was determined from:

$$\Delta G^\circ = -RT \ln K_p \quad (3)$$

The enthalpy change ( $\Delta H^\circ$ ) was obtained from the van't Hoff equation:

$$\ln \frac{K_{pT_2}}{K_{pT_1}} = \frac{\Delta H^\circ}{R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right) \quad (4)$$

in which  $K_{pT_2}$  and  $K_{pT_1}$  are the partition coefficients determined at two different temperatures. Three  $\Delta H^\circ$  values were calculated by combining the assayed temperatures ( $T_1 = 5$  °C;  $T_2 = 20$  °C and  $T_3 = 37$  °C) and the corresponding Kps in pairs. Similar  $\Delta H^\circ$  values were obtained, thus indicating this function maintained constant at the working temperature range. The mean value was then informed.

Finally, the entropy change ( $\Delta S^\circ$ ) was calculated from:

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \quad (5)$$

**2.2.1.4. Measurement of enzyme activity.** Phospholipase  $A_2$  activity was evidenced by the formation of hemolytic halos in agarose-erythrocyte egg yolk gels. This indirect hemolytic activity was estimated according to the method described by Gutiérrez et al. [26]. Three hundred micro-liters of packed sheep erythrocytes were washed with saline solution. Then, 300  $\mu\text{L}$  of 1:3 egg yolk solution in saline solution and 250  $\mu\text{L}$  of 0.01 M  $\text{CaCl}_2$  solution was added to 25 mL of 1% (wt/v) of agar (at 50 °C) dissolved in PBS pH 7.2. The mixture was applied to plastic plates (135  $\times$  80 mm) until gelling. Aliquots (15  $\mu\text{L}$ ) of phase (top/bottom) from partitioning experiments were applied into 3 mm-diameter wells in the centre of the gel. After 20 h of incubation at 37 °C, the diameters of hemolytic halos ( $D_{\text{halo}}$ /mm) were measured. The  $\text{PLA}_2$  concentration ( $[\text{PLA}_2]$ /mg/mL) was calculated from the expression:

$$[\text{PLA}_2] = e^{\left( \frac{D_{\text{halo}} - 21.324}{2.2989} \right)} \quad (6)$$

This relationship was obtained from a  $D_{\text{halo}}$  vs.  $[\text{PLA}_2]$  curve obtained in our laboratory by filling the wells with different amounts of isolated  $\text{PLA}_2$ . Assays with PBS and top/phase (without venom) were also carried out in parallel as blanks.

Proteolytic activity was determined by the modified colorimetric method, described by Wang and Huang [27]. An aliquot (45  $\mu\text{L}$ ) of the phase from partitioning experiment was added to 255  $\mu\text{L}$  of an azocasein solution (5 mg/mL) in phosphate buffer saline (pH 7.2). The mixture was incubated at 37 °C for 90 min and

then, the reaction was stopped by the addition of 5% trichloroacetic acid (600  $\mu\text{L}$ ). After letting stand at room temperature for 30 min, the mixture was centrifuged at 3000 rpm for 5 min. The supernatant was mixed with an equal volume of 0.5 M NaOH and the absorbance at 450 nm was determined. Proteolytic activity was expressed as the change in absorbance measurements. Blanks were prepared with phase (top/bottom) in absence of venom.

The concentration of total protein (TP) was determined according to the Bradford method [28], based on the change in the absorption spectrum of the Coomassie blue dye when it binds to proteins under acid conditions. A pooled freeze-dried venom was used as standard since the response depends on the amino-acid composition of the protein. In order to take into account the possible polyethyleneglycol–dye interaction, several calibration curves were obtained by mixing the Coomassie blue reactive with known quantities of the standard venom in solutions of the different assayed PEGs (PEG1000, PEG3350, PEG6000 and PEG8000).

The absorbance measurements at 595 nm were carried out on a Boeco S-22 UV/visible Spectrophotometer (Germany).

**2.2.1.5. Performance parameters of the liquid–liquid extraction.** The recoveries in percentage of target enzymes ( $R_{\text{PLA}_2}$ ,  $R_p$ ) at the top/bottom phase were calculated according to the following expression:

$$R_{\text{enzyme, top/bottom}} (\%) = 100 \frac{\text{Act}_{\text{top/bottom}} V_{\text{top/bottom}}}{\text{Act}_{\text{initial}} V_{\text{initial}}} \quad (7)$$

where  $\text{Act}_{\text{top/bottom}}$  represents the enzyme activity in a given phase (top/bottom) and  $\text{Act}_{\text{initial}}$  represents the enzyme activity in the venom suspension loaded into the aqueous two-phase system. The  $V_{\text{top/bottom}}$  and  $V_{\text{initial}}$  are the volumes of the phases and the volume of added venom suspension respectively.

The purification factor ( $\text{PF}_{\text{PLA}_2}$ ,  $\text{PF}_p$ ) in each phase was determined as follows:

$$\text{PF}_{\text{enzyme, top/bottom}} = \frac{\text{Act}_{\text{top/bottom}} / [\text{TP}]_{\text{top/bottom}}}{\text{Act}_{\text{initial}} / [\text{TP}]_{\text{initial}}} \quad (8)$$

where  $[\text{TP}]_{\text{top/bottom}}$  and  $[\text{TP}]_{\text{initial}}$  are the total protein (TP) concentration in the phase and in the venom suspension respectively.

The mass balance (MB) in percentage was calculated in each case to evaluate the precipitation of the target enzymes/total proteins at the interface according to:

$$\text{MB} (\%) = 100 \frac{\text{Act}_{\text{top}} V_{\text{top}} + \text{Act}_{\text{bottom}} V_{\text{bottom}}}{\text{Act}_{\text{initial}} V_{\text{initial}}} \quad (9)$$

When calculating the mass balance of total protein, the total protein concentration ( $[\text{TP}]$ ) replaced the activity values (Act) in the above expression.

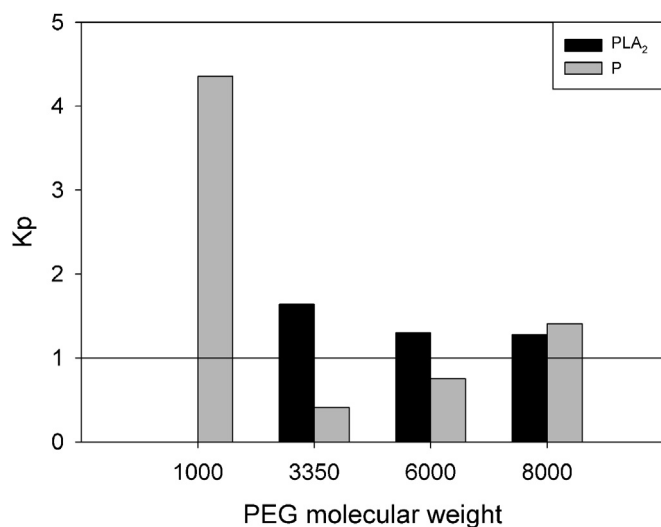
**2.2.1.6. Batch adsorption with DEAE cellulose.** Equal volumes of top phase (PEG-enriched) from venom partitioning and a DEAE cellulose suspension (in buffer 10 mM KPi pH 7.0) were mixed. A thorough gentle mixing of the system was carried out for 1 h to favour the interaction between the resin and the proteins. A further low speed centrifugation was used to separate the insoluble cationic polyelectrolyte (DEAE-cellulose) from the supernatant. After washing the resin with KPi buffer pH 7.0, the elution was achieved by gradually increasing ionic strength of the buffer via salt gradient until 1 M NaCl (in buffer Pi 10 mM pH 7.0). Measurements of  $\text{PLA}_2$  activity and the total protein concentration were used to follow the process.

### 3. Results and discussion

#### 3.1. Partitioning pattern of PLA<sub>2</sub> and P. Thermodynamic characterization

The partitioning pattern of PLA<sub>2</sub> and P in aqueous two-phase systems prepared with PEGs of different molecular weights was evaluated and compared. From a visual inspection of Fig. 1, two apparently opposite trends are observed for PLA<sub>2</sub> and P partitioning when increasing the size of PEG molecule. On one hand, the increase in PEG molecular weight decreased the  $K_{p,PLA_2}$ , this effect being more pronounced for PEGs of lower molecular weight. Notice that the  $K_{p,PLA_2}$  corresponding to PEG1000 ATPS was not achieved (see Fig. 1) since the PLA<sub>2</sub> content in the bottom phase of this system was too low to be detected by the hemolytic quantifying method of the enzyme. This indicates that PLA<sub>2</sub> is unevenly distributed to the top polymer enriched-phase ( $K_p \approx \infty$ ). On the other hand, P shows an initial decrease in  $K_p$  when PEG MW increases from 1000 to 3350 and a later increase in this parameter ( $K_p$ ) when PEG MW varies from 3350 to 8000. This reflects different driving forces involved in the partitioning behaviour of PLA<sub>2</sub> and P.

It is well-known fact that the partition coefficient ( $K_p$ ) depends on a broad array of factors such as the structure and MW of the phase forming polymers, the pH, salt concentration, the MW and hydrophobicity of the partitioned molecule [15]. To our knowledge, no state-equation that explains completely the relationship between the partition coefficient and these factors has been developed yet; however, partial explanations are available. Several approaches, derived by Johansson [29] stated explicitly the dependence of  $K_p$  on the enthalpy and entropy forces. According to these treatments, a decrease in the  $K_p$  value with the reciprocal of PEG molecular weight is an expectable trend when the entropy contribution prevails [30]. This is a consequence of an exclusion effect and the consequent reduction of the available volume to proteins in the polymer phase (top phase) when the PEG size increases. This seems to be an appropriate explanation for the decrease in  $K_{p,PLA_2}$  in systems with PEG MW increasing from 1000 to 8,000, but only accounts for the P behaviour in systems with PEG MW from 1000 to 3350. The later increase in  $K_{p,P}$  for ATPSs formed by larger PEGs would evidence increasing enthalpy contributions derived from the interaction between the protein (solute) and the



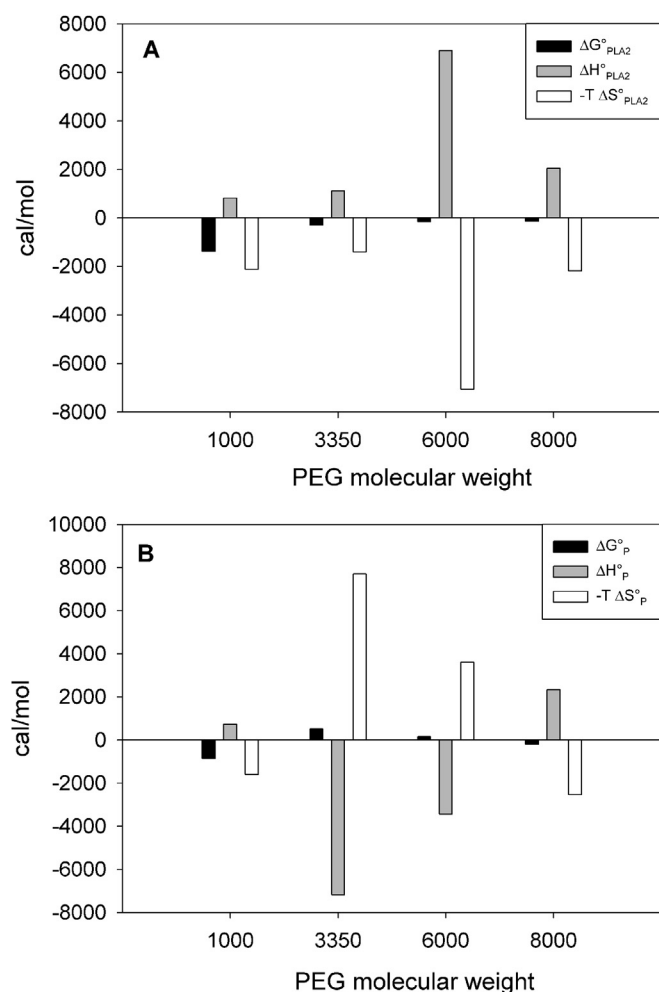
**Fig. 1.** Effect of PEG molecular weight on the partition coefficient ( $K_p$ ) of Phospholipase A2 (PLA<sub>2</sub>) and protease (P) of *Bothrops alternatus* venom. Temperature: 293K. Total system compositions are those of Table 1.

phase-polymer.

The thermodynamic parameters associated to the partitioning equilibrium of PLA<sub>2</sub> and P were calculated and represented in Fig. 2. A transfer mechanism –from bottom to top phase– governed by a prevalent entropy contribution ( $-T \Delta S^\circ < 0$ ) is observed for PLA<sub>2</sub> in all the assayed systems and for P in those systems formed with the lighter and heavier PEG (PEG1000 and PEG8000). Positive enthalpy changes in these cases compensate the entropy changes, thus evidencing the presence of a hydrophobic effect which involves structuring/destructuring of water molecules surrounding the hydrophobic surface areas of PEG and protein molecule. This trend has also been reported previously for the partitioning of several biomolecules [30,31]. Negative enthalpy changes associated to the partitioning equilibrium of P in systems formed by PEG of intermediate sizes (PEG3350 and PEG6000) indicates the prevalence of electrostatic forces in the P partitioning. It should be kept in mind that venom is a complex mixture of different biological components and therefore, the interactions among these components could play a crucial role in determining partitioning behaviour.

#### 3.2. Extraction performance: effect of PEG molecular weight, volume ratio ( $V_{top}/V_{bottom}$ ) and venom load

The purification parameters associated to the extraction of PLA<sub>2</sub>



**Fig. 2.** Thermodynamic parameters associated to the transfer of PLA<sub>2</sub> and P to the top phase of aqueous two-phase systems formed by potassium phosphate and PEGs of different molecular weight. Temperature: 293K. Total system compositions are those of Table 1. (A) PLA<sub>2</sub>, (B) P.

and P toxins from *B. alternatus* venom were calculated (Fig. 3). According to the partitioning pattern analysed previously, PLA<sub>2</sub> and P were expected to be recovered mainly at the top and bottom phases respectively. Therefore, the R (%) and the PF in each phase were informed. The recovery pattern of PLA<sub>2</sub> was observed to follow the sequence predicted by its partitioning coefficients (Fig. 1). This enzyme was practically recovered (50–72%) in the top phase of all the assayed systems ( $K_{p,PLA_2} > 1$ ), this recovery decreasing with PEG size. Values of R (%) lower than 40% were obtained at the bottom phase (data not shown). The mass balance resulted to be between 92 and 103% for all the systems. This indicated that no significant precipitation of PLA<sub>2</sub> took place at the interface.

The trend observed for the recovery of P at the bottom phase was also in agreement with its partitioning behaviour. An initial increasing in R (%) when changing PEG molecular weight from 1000 to 3350 was observed. A further decrease in R (%) was observed for systems formed by larger PEGs. The highest R value (82%) corresponded to the lowest  $K_{p,P}$  (0.41) obtained for the PEG3350/KPi ATPS. However, unexpected too low recoveries were obtained for the other systems (10–28%). For these systems, a faint precipitate formed at the interface when the whole venom was loaded into the systems. Mass balances calculated in these cases were from 60 to 70%, thus indicating a loss of P at the interface. The interactions between proteins and phase components may modify the structure and biological function of macromolecules, and consequently,

affect their partition behaviour. In this context, the precipitation behaviour observed for P could be derived from the labile nature of this enzyme and its higher susceptibility to suffer conformational changes [32].

When analysing the PF values, it was observed that the PEG3350/KPi ATPS exhibited the best performance in purifying PLA<sub>2</sub> and P at the top and at the bottom phase respectively. A 2-fold purification, obtained for PLA<sub>2</sub> at the top phase, could be attributable to the selective transfer of P to the bottom phase. On the other hand, no significant purification (PF 1.2) was obtained for P. In this case, the presence of PLA<sub>2</sub> (approximately 30%) and the rest of venom proteins (close to 40%) at the bottom phase would be responsible of this poor performance.

Despite of these results, it should be addressed that the bottom phase (after extraction) is depleted in PLA<sub>2</sub> when comparing with the whole venom. If the rest of proteins, apart from PLA<sub>2</sub> and P, were removed, an extract mainly composed by PLA<sub>2</sub> and P would be achievable. According to a previous work [33], a multi-step extraction strategy followed by a gel filtration chromatography was demonstrated to be an efficient alternative to recover P of high purity.

The remaining parameters that affect the extraction process are the volume ratio ( $V_r = V_{top}/V_{bottom}$ ) and the venom load. They only were evaluated in PEG3350/KPi ATPSs since these systems exhibited the best purification performance. If no precipitation at the interface took place, the relationship between  $R_{top}$  and  $V_r$  would be:

$$R_{top}(\%) = \frac{K_p V_r}{1 + K_p V_r} 100 \quad (10)$$

Clearly, the above expression predicts an increase in the  $R_{top}$  (%) at higher volume ratios. Conversely, the  $R_{bottom}$  would be expected to decrease according to:

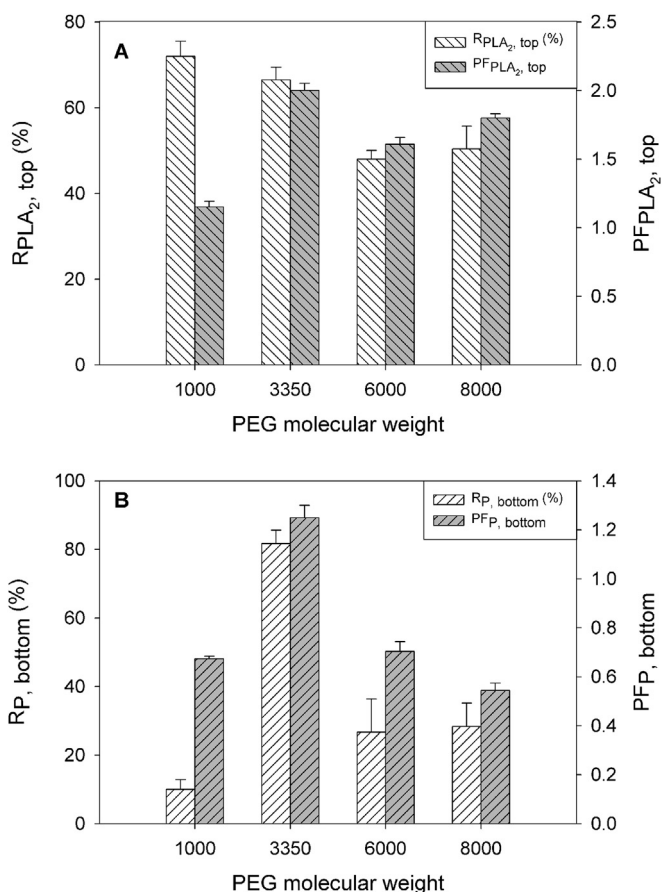
$$R_{bottom}(\%) = \frac{1}{1 + K_p V_r} 100 \quad (11)$$

The effect of  $V_r$  on  $K_p$ , R (%) and PF values was also analysed (Fig. 4). It is addressed that the partitioning behaviour changes with volume ratio similarly it was observed in other systems [34,23]. When reducing the volume of a given phase respect to the other in order to modify the  $V_r$ , that phase becomes more concentrated in venom components and the interactions among them change. This affects the activity coefficient of the target molecule and modifies its partition coefficient and recovery. The yield of PLA<sub>2</sub> at the top phase is observed to rise when increasing the volume ratio from 0.5 to 2 (Fig. 4). When comparing the experimental recoveries with those calculated with Eq. (10), no significant differences were observed. For P, however, the experimental recoveries (bottom phase) at extreme volume ratios (i.e. 0.5 and 2) are significantly lower than those calculated with Eq. (11). In this case, the decrease in phase volume not only caused changes in interactions but produced the precipitation of the target protein (P). This fact agreed with the observation of a precipitate at the interface and was later confirmed by the calculated mass balance (40–60%).

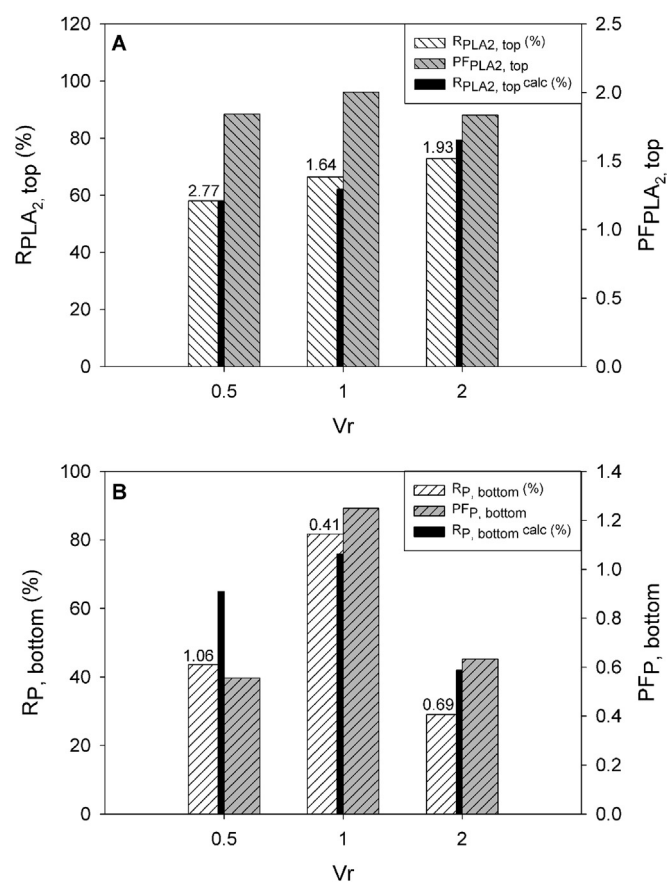
The effect of volume ratio on PF was also analysed. By either reducing or increasing this parameter ( $V_r$ ), no enhancement in PF was achieved.

The best performance, i.e. higher PF for PLA<sub>2</sub> and P in top and bottom phase respectively, was observed for the system with equal phase volumes. Changes in the interactions among phase components or precipitation did not reflex in a selectivity improvement.

Finally, the effect of venom load on the performance of extraction process was assayed (Fig. 5). Different trends were observed for PLA<sub>2</sub> and P. A significant decrease in both recovery and PF of PLA<sub>2</sub> is



**Fig. 3.** Effect of PEG molecular weight on the purification parameters associated to the extraction of PLA<sub>2</sub> and P from *Bothrops alternatus* venom with ATPS. Temperature 20 °C,  $V_{top}/V_{bottom} = 1$ . Total system compositions are those of Table 1. (A) PLA<sub>2</sub> at the top phase, (B) P at the bottom phase. Each bar represents the mean value of three determinations and its standard deviation.



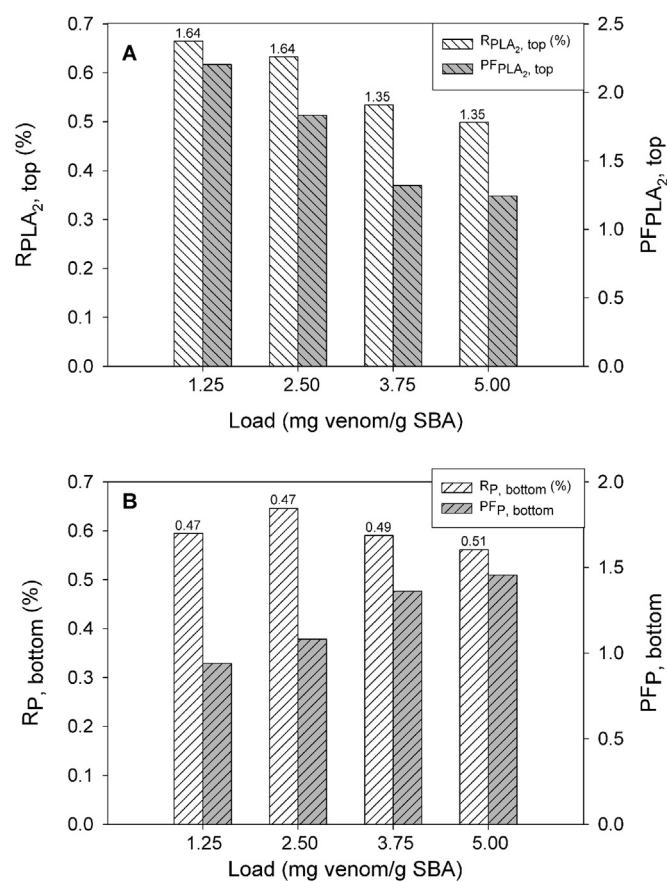
**Fig. 4.** Effect of volume ratio on the purification parameters associated to the extraction of PLA<sub>2</sub> and P from *Bothrops alternatus* venom. The black bars represent the recoveries calculated with Eqs. (10) and (11). Temperature 20 °C, PEG molecular weight 3350. Total system compositions are those of Table 1. Numbers above the bars represent the Kp values obtained in each case. (A) PLA<sub>2</sub> at the top phase, (B) P at the bottom phase.

observed when increasing the venom loaded into the SBA. This could be related to the decrease in Kp<sub>PLA<sub>2</sub></sub> from 1.64 to 1.35 and the precipitation of the enzyme at the interface. Both features probably derive from the existence of a solubility limit of PLA<sub>2</sub> in the top phase (enriched in PEG), which is not able to be overcome with higher loads. This was evidenced when calculated the mass balance for this enzyme since it came down from 1.00 to 0.86.

On the other hand, Kp values and recoveries of P (in the bottom phase) were practically unaffected by the venom load. Nevertheless, the PF was slightly enhanced from 1.25 to 1.45, this being attributable to the loss of PLA<sub>2</sub> at the interface since this toxin represents one of the main contaminants at the bottom phase.

### 3.3. Removing PEG

As it was stated above, an extract enriched in PLA<sub>2</sub> and depleted in P can be obtained from the top phase of a PEG3350/KPi SBA. According to the phase compositions shown in Table 1, this extract will be concentrated in PEG polymer (23.90% wt/wt). Although the biocompatibility and the non-immunogenicity of the PEG molecule its high content in a mixture could make difficult a further manipulation and lyophilisation procedure. Therefore, a step for removing the PEG was assayed. Taking into account the acidic character of PLA<sub>2</sub>, we found that a simple batch adsorption with the cationic exchange resin DEAE cellulose was able to extract practically the totality of PLA<sub>2</sub> present in the top phase of SBA. A further



**Fig. 5.** Effect of venom load on the purification parameters associated to the extraction of PLA<sub>2</sub> and P from *Bothrops alternatus* venom. Temperature 20 °C, PEG molecular weight 3350. Total system compositions are those of Table 1. Numbers above the bars represent the Kp values obtained in each case. (A) PLA<sub>2</sub> at the top phase, (B) P at the bottom phase.

elution with buffer Pi 10 mM pH 7.0 and NaCl 0.25 M was required to recover the active enzyme, free of polymer. This extract was easily lyophilised and stored at –20 °C until using.

## 4. Conclusions

In this work we explored the possible application of liquid–liquid extraction with aqueous two phase systems to recover mixtures enriched in the main toxins of *B. alternatus* venom. After evaluating different parameters such as the polymer size, the volume ratio and the venom load, we demonstrated that PEG3350/KPi pH 7.0 aqueous two-phase systems (with equal phase volumes and a load of 1.25 mg of venom per g of system) were capable of distributing selectively the PLA<sub>2</sub> and P, main venom toxins, into the top and bottom phase respectively. The differential partition behaviour of these enzymes was observed to be consequence of opposite thermodynamic forces involved in the distribution between phases, either entropically- or enthalpically-driven mechanisms being prevalent in each case.

As the result, a top extract, enriched in PLA<sub>2</sub>, was obtained. An additional adsorption step with DEAE cellulose demonstrated to be effective to remove the PEG polymer from the top phase and recover the active PLA<sub>2</sub>. The P was mainly recovered in the bottom phase with a content of PLA<sub>2</sub> lower than that present in the whole venom. According to previous reports, these venom mixtures enriched either in PLA<sub>2</sub> or P could be capable of being used in milder toxic immunizing protocols to obtain specific antisera. The

proposed procedure is simply, inexpensive and only requires professionals trained in handling basic laboratory equipment. It could be easily adopted by countries with scarce resources, in which the snakebite accidents are considered a public health concern.

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